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Spread of Hepatitis B Viruses In Vitro Requires Extracellular Progeny and May Be Codetermined by Polarized Egress

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Viruses can spread by different mechanisms: via intracellular particles through cell junctions to neighboring cells or via secreted virions to adjacent or remote cells. The observation of clusters of hepadnavirus-infected cells both in vivo and in primary hepatocytes neither proves the first mechanism nor excludes the second. In order to test which mechanism, if not both, is used by hepatitis B viruses in order to spread, we used primary duck hepatocytes and duck hepatitis B virus (DHBV) as an infection model. If extracellular progeny virus alone determines spreading, neutralizing antisera or drugs blocking virus binding to hepatocytes should abolish secondary infection. In order to test this, we used DHBV envelope-specific neutralizing antisera, as well as suramin, a known inhibitor of infection. Both reagents strongly reduced hepatocellular attachment of viral particles and almost completely abolished primary infection, whereas an ongoing intracellular infection was not affected as long as no progeny virus was released. In contrast, incubation of infected primary hepatocytes with these reagents during release of progeny virus completely prevented secondary infection. Moreover, the combination of electron and immunofluorescence microscopy analyses revealed the residence of viral particles in cytoplasmic vesicles preferentially located near the basolateral membrane of infected hepatocytes. Taken together, these data strongly suggest that hepatitis B viruses mainly spread by secreted, extracellular progeny and point to polarized egress of viral particles into intercellular compartments, which restricts their diffusion and favors transmission of virus to adjacent cells.

Hepadnaviruses, including the prototype human hepatitis B virus (HBV), are small enveloped DNA viruses that predominantly replicate in hepatocytes in a noncytotoxic manner. Upon exposure of ducklings to duck HBV (DHBV) at a low multiplicity of infection, virtually all hepatocytes in the liver tissue are infected within a short time period (6). Given the enormous size of the liver, with ca. 10^{11} hepatocytes, the mode of viral spread must therefore be very efficient. In vivo, clusters of virus-replicating cells are frequently observed during the early phases of infection (5). Moreover, similar clusters are also seen in primary hepatocyte cultures (unpublished data). These seminal observations indirectly suggest that HBVs are transmitted from cell to cell and may indicate that the infectious movement of progeny virus in the extracellular environment is not controlled by diffusion alone. On the other hand, it is well known that a large number of virions, as well as noninfectious subviral particles lacking nucleocapsids, are secreted from infected hepatocytes into the extracellular space, where free diffusion should allow infection of remote host cells.

Alphaherpesviruses, human immunodeficiency viruses (HIV), and poxviruses are able to move from an infected cell to an adjoining uninfected cell by direct cell-to-cell spread (3, 13, 16). This transmission occurs specifically at sites of cell-cell contact; herpesviruses, for example, move to neurons across epithelial cells or neuronal junctions. This type of viral spread is typically very rapid and efficient; this is not only due to the close spatial proximity of virus and cellular determinants of infection but may also be due to the protection of progeny virus from neutralizing antibodies or other immune system components by junctions. Other viruses exploit different modes of spread that also proved to be very efficient. Vaccinia virus, for example, spreads by at least two modes, one of which protects the virus from neutralizing antibodies (suggesting direct spread), whereas the other is antibody sensitive (suggesting spread through extracellular virus) (9).

In the present study, we analyzed the mechanism of hepadnaviral spread in vitro by using DHBV and primary duck hepatocytes (PDHs) as a model system. To date, it is unknown whether HBVs are transmitted directly from cell to cell or if infection of neighboring cells requires progeny first to be released from the producer cell. In the latter case, virus infection should be sensitive to neutralizing antibodies and drugs that interfere with the binding of viral particles to their target cells. By using two neutralizing antisera (2, 15) and suramin, a drug known to abolish DHBV infection of hepatocytes (11), we obtained evidence that infection of PDHs during the second round is mainly, if not exclusively, achieved by progeny virus secreted into the extracellular space. Furthermore, we provide evidence that the egress of DHBV particles is polarized, which may at least partially contribute to the preferential transmission of virus to adjacent cells.

MATERIALS AND METHODS

Primary hepatocyte cultures and cell lines. PDHs were prepared and cultivated as described elsewhere (14). Briefly, PDHs were prepared from livers of fetal ducks by the collagenase digestion method. Liver cells were resuspended in Williams' medium E (Gibco-BRL) supplemented with 1.5% dimethyl sulfoxide, 1 nM insulin, and 10 μM hydrocortisone (all from Sigma, Steinheim, Germany),

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2 mM glutamine, 15 mM HEPES (pH 7.2), 100 U of penicillin/ml, and 100 μg of streptomycin/ml and then seeded into 12-well plates at a density of ca. 5×10^5 liver cells per well. Persistently DHBV-replicating D2 cells were maintained in Dulbecco's modified Eagle's medium containing 2 mM glutamine, sodium pyruvate, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% fetal calf serum. All cells were grown in a humidified 5% CO_2-air atmosphere.

Viruses, antibodies, and drugs. PDHs were infected with a DHBV-viremic goose serum containing 1.1×10^{10} genome equivalents (GE)/ml as determined by dot blot analysis. Serum pools were divided into aliquots and stored at −70°C. For attachment and infection assays, we used two rabbit anti-DHBV preS-specific sera (designated DPSI and KpnI), an unrelated control rabbit serum, and immunopurified rabbit anti-mouse immunoglobulin G (IgG; Sigma). The DPSI and KpnI antibodies were raised against recombinant DHBV L protein spanning amino acids 1 to 131 and amino acids 44 to 185 of the preS domain, respectively (4, 15). Suramin was purchased from Sigma, dissolved in dimethyl sulfoxide (stock of 100 mg/ml) and stored at −20°C. For immunoblot and fluorescence analyses, we used polyclonal rabbit anti-DHBV preS serum as described previously (14). Secondary, goat anti-rabbit antibodies conjugated with Alexa 488 or horseradish peroxidase were obtained from Molecular Probes (Leiden, The Netherlands) and Dianova (Hamburg, Germany), respectively.

Attachment and interference assay. To determine the amount of virions bound to the cell surface of PDHs, cultures were first pretreated for 0.5 h with 100 μg of suramin/ml or were left untreated. They were then inoculated with viremic goose serum corresponding to 220 GE per hepatocyte. Thereafter, cells were transferred to 4°C for 2 h to allow virus binding but not uptake. After an extensive washing with phosphate-buffered saline (PBS), the cells were harvested by lysis in PCR sample buffer (8). To investigate the effect of neutralizing antibodies on viral attachment, the inoculum was preincubated with 10 μl of the antisera DPSI, KpnI, control rabbit serum, or 20 μg of purified IgG for 0.5 h at 37°C and then added to the cells. Cultures were incubated for 2 h at 4°C before they were extensively washed with PBS and harvested in 200 μl of PCR sample buffer. For analysis of the GE dependency of viral attachment, PDHs were inoculated with different amounts of viremic serum corresponding to 2, 22, 110, and 220 GE/cell; the PDHs were then incubated and harvested as described above.

Detection of viral DNA by PCR. Each sample was digested for 2 h at 56°C with proteinase K (Roche, Mannheim, Germany); the enzyme was then inactivated for 10 min at 95°C, and the sample was subjected to PCR with DHBV-specific primers as described previously (8). For semiquantitative PCR analysis, 10-fold serial dilutions of a viremic serum with known GE were included in each PCR run.

Inhibition of primary infection. To investigate the effects of the antisera and suramin on primary infection, viremic serum was preincubated with the same amount of antisera as described above or the cells were pretreated with suramin (pretreatment) before inoculation of cells. Alternatively, 100 μg of suramin or 10 μl of DPSI antiserum/ml was added to the cells along with the inoculum (synchronous treatment) or after 2 h (posttreatment). Subsequently, cells were incubated overnight at 37°C, and thereafter unbound viral particles were removed by washing the cells. Bound, not-yet-internalized virions were inactivated by a low-pH shock (8). The cells were further incubated for 3 days before harvest and then analyzed for preS protein by immunostaining and blotting.

Inhibition of secondary infection and viral spread. PDHs were infected with 220 GE/cell overnight; medium was then changed, and 3 days later the cells were incubated with either 100 μg of suramin or 10 μl of antiserum DPSI/ml. At this time point, the infected cells start to secrete progeny virus. The medium was renewed daily, and fresh substances were added. Three days later, cells were fixed and stained as described below.

Effect of suramin and neutralizing preS antisera on ongoing viral replication. To assay the effect of antisera and suramin on viral replication and secretion, the persistently DHBV-propagating LMH cell line D2 was incubated continuously for 3 days with the same amount of suramin and preS antiserum as used for the inhibition of secondary infection, and then cell culture supernatants and cells were harvested and subjected to PCR or immunoblot analysis of viral DNA or preS protein, respectively.

Confocal laser scanning microscopy. PDHs grown on coverslips were infected with DHBV; 1 week later they were fixed and stained for preS. Pictures were acquired with a scanning confocal microscope (LSM 510 META; Zeiss, Berlin, Germany) and processed by using the Openlab software.

Electron microscopy. Chronically infected PDHs were fixed with 2.5% glutaraldehyde in PBS for 20 min at room temperature, washed, and postfixed for 30 min with 1% OsO_4 in PBS. For ultrathin sectioning, the samples were gradually dehydrated with ethanol and embedded in ERL resin. Sections were counterstained with 2% uranyl acetate and lead citrate. The electron micrographs were obtained with a Philips CM 120 transmission electron microscope at 60 kV.

Immunofluorescence microscopy. Cultures were washed once with PBS and fixed with an ice-cold mixture of methanol-acetone (1:1) for 10 min at room temperature. Cells were rehydrated with PBS and then incubated for 1 h with rabbit anti-DHBV preS serum KpnI diluted 1:800. Cells were then washed three times with PBS and incubated with secondary goat anti-rabbit Alexa 488-labeled (green) antibody (diluted 1:800) for another 30 min. Nuclei were counterstained with Hoechst (final concentration, 4 μg/ml). After they were mounted and embedded, stained cells were analyzed and photographed exactly as described previously (14).

SDS-PAGE and immunoblotting. Cells grown in 12-well culture plates were washed with PBS and directly lysed in 200 μl of 4× Laemmli buffer per well. All samples were boiled at 99°C for 5 min, and 20 μl of each sample was fractionated by denaturating sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, proteins were transferred onto nitrocellulose membranes. After they were blocked with 3% dry milk diluted in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl), the membranes were incubated for 1 h at room temperature with rabbit anti-DHBV preS antiserum KpnI (diluted 1:20,000). After several washes with TBS plus 0.1% Tween 20, membranes were further incubated with horseradish peroxidase-coupled goat anti-rabbit IgG antibodies at a dilution of 1:20,000. Proteins were visualized by enhanced indirect chemiluminescence (Pierce).

RESULTS AND DISCUSSION

The spread of viruses via extracellular progeny is sensitive to neutralizing antibodies or substances that interfere with the initial steps of virus-cell interaction (9). In order to identify substances that specifically prevent the attachment of DHBV particles to hepatocytes that are required to elucidate the spreading mode of DHBV, we tested the effect of suramin and two different preS-directed antisera on the attachment of DHBV to PDHs. Suramin was previously reported to prevent DHBV infection of hepatocytes by interference with an as-yet-unspecified early infection step (11, 12). Since suramin prevents binding of HIV and herpesviruses to host cells (1, 17), we assumed that the same applies to DHBV. The preS domain of the L protein has been shown to mediate interaction of DHBV to hepatocytes, and antibodies binding to this domain are expected to neutralize virus infection by interference with attachment of virions to host cells. To test this experimentally, we used two independent DHBV L-specific antisera directed against the preS domain (DPSI and KpnI; see Materials and Methods) in our attachment interference assay. As a specificity control for the anti-preS antibodies, we used immunopurified mouse anti-rabbit IgG and unrelated rabbit antiserum.

To determine the effect of suramin and the preS-specific antisera on attachment of DHBV to hepatocytes, cells were pretreated for 0.5 h with 100 μg of suramin/ml or viremic serum preincubated with anti-preS sera or control sera prior to inoculation of the cells at 4°C for 2 h. The experimental conditions used allow only virus binding to but not uptake into the hepatocytes. Subsequently, cells were washed and harvested, and the amounts of cell-associated viral DNA were determined after agarose gel electrophoresis of amplified PCR products. Tenfold serial dilutions of a DHBV viremic serum with known GE were included to calibrate the PCR (Fig. 1A, lanes 11 to 14). These experiments revealed an assay sensitivity of $\sim 10^4$ GE and linearity between 10^4 and 10^6 GE. The amount of attached virions proportionally increased with more GE per cell used (2 to 220) (Fig. 1A, lanes 1 to 5). The two neutralizing anti-preS antibodies used strongly inhibited the attachment of virions to PDHs (Fig. 1A, lanes 5 to 7). This was specific since

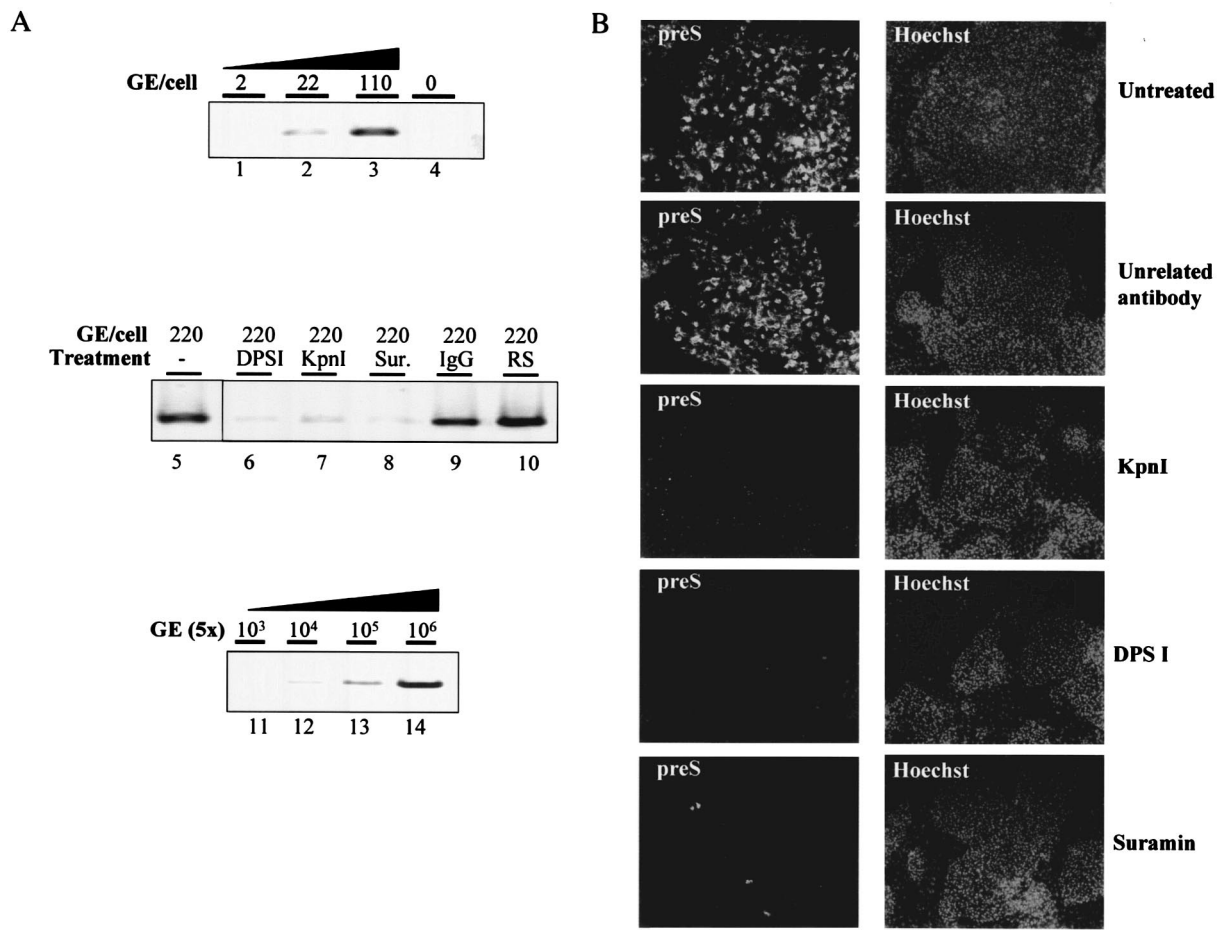

FIG. 1. (A, upper panel) Hepatocellular attachment of DHBV increases with larger amounts of GE. To analyze the GE dependency of virus binding to hepatocytes, cells were incubated with DHBV at different GE levels for 2 h at 4°C, washed, harvested, and subjected to DHBV DNA-specific PCR. (A, lower panel) preS-specific antisera DPSI and KpnI, as well as suramin, impair viral attachment to hepatocytes. DHBV viremic serum or cells were preincubated for 0.5 h at 37°C with the indicated antisera or suramin, respectively. Subsequently, the cultures were inoculated and shifted to 4°C for 2 h to allow virus binding to cells. Cultures were then washed to remove unbound viral particles and then harvested. Next, the amount of cell-associated viral DNA was determined after agarose gel electrophoresis of amplified PCR products. Tenfold serial dilutions of a DHBV viremic serum with a known GE level were included to calibrate the PCR. Sur., suramin; RS, nonspecific control rabbit serum. (B) Diminished virion binding leads to reduced infection. Cultures treated and inoculated as described above were further cultivated for 3 days, fixed, and immunostained for preS protein, indicating successful viral infection. Nuclei were counterstained with Hoechst.

the preincubation of inoculum with nonspecific IgG or control rabbit serum (Fig. 1A, lanes 5, 9, and 10) had no effect. Suramin reduced binding of DNA-containing particles to hepatocytes to an extent similar to that of the neutralizing antibodies (Fig. 1A, lanes 5 to 8), indicating that the antiviral effect of suramin is mainly due to its interference with virus attachment. In order to correlate the results obtained from the attachment interference assays with productive infection, treated cultures were incubated for 3 days and stained for preS as an indicator for successful infection. It was obvious that the reduced binding resulted in a dramatically reduced number of infected cells in both the anti-preS serum-treated and the suramin-treated cultures (Fig. 1B). In contrast, the unrelated antiserum that did not reduce viral binding also had no influence on infection efficiency (Fig. 1B, unrelated antibody). Taken together, these findings imply that most of the virus binding measured under our experimental conditions is specific and relevant for productive infection.

In order to determine any possible effects of suramin or the anti-preS sera postattachment, we investigated whether preS antiserum and suramin affect productive primary infection when given simultaneously or after virus attachment and, as a further control, also when given before attachment. DHBV viremic serum and PDHs were preincubated with the antibody and suramin, respectively, and then the inocula were added to the cells. Alternatively, suramin and DPSI antibody were added to the cells together with the inoculum or 2 h later. Expression of preS protein was measured 3 days later and used as an indicator for productive infection. Immunoblot analysis of the cellular lysates showed that suramin and anti-preS serum DPSI strongly reduced infection when they were administered prior to or concomitantly with virus to the cells (Fig. 2A, lanes 2, 3, 4, 6, and 7). The addition of antibody or drug 2 h after exposure of the cells to virus had no effect on the efficiency of productive DHBV infection compared to untreated cells (Fig. 2A, lanes 2, 5, and 8). Taken together, both reagents

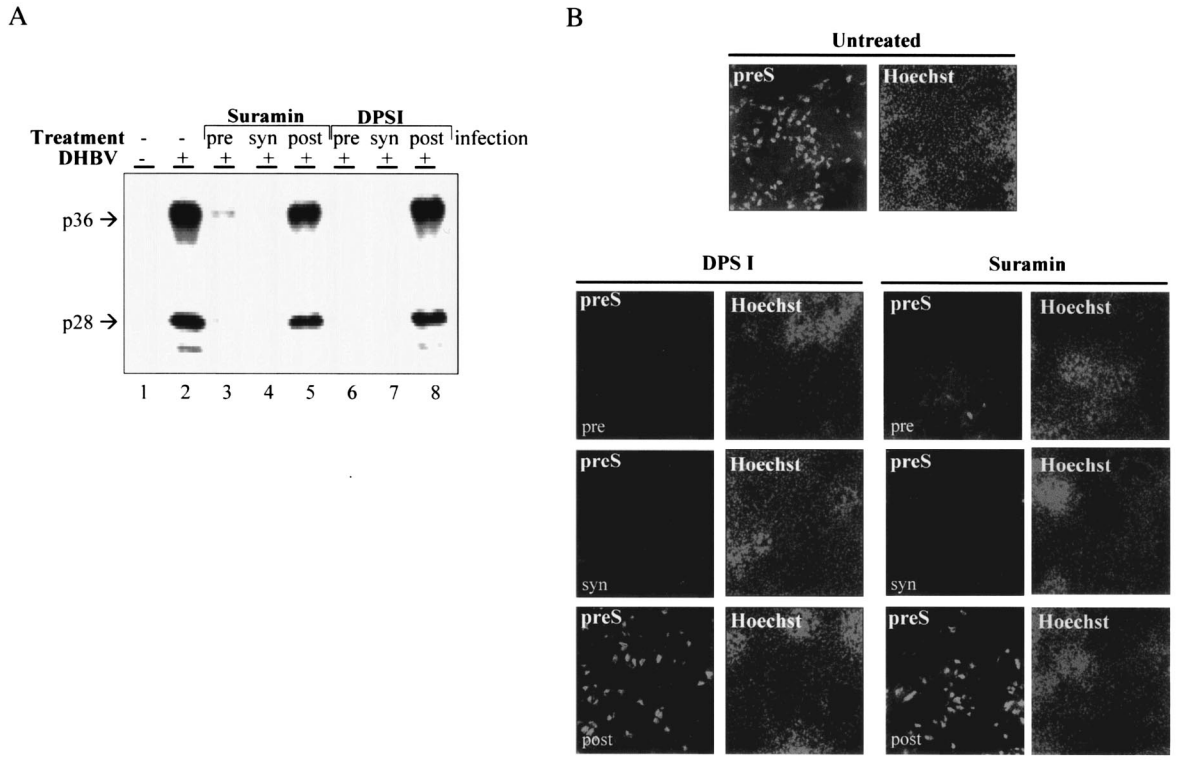

FIG. 2. Productive infection is diminished in antibody- or suramin-treated cells when they are treated prior to infection or simultaneously with inoculation. (A) preS immunoblot analysis of cell lysates. PDHs and inoculum were either pretreated or preincubated with suramin or antiserum DPSI, respectively (pre), or the substances were given along with the inoculum (syn) or postinoculation (post). Three days after infection, the cells were harvested in Laemmli buffer and subjected to denaturating SDS-PAGE. Arrows point to the p36 and p28 form of the DHBV L protein. (B) Corresponding immunofluorescence staining of cells treated as described in panel A. Cells were fixed and immunostained for preS protein expression (preS), and nuclei were counterstained with Hoechst.

have no detectable postattachment effects on primary infection and thus are optimally suited for deciphering the mechanisms of hepadnaviral spreading. Suramin strongly inhibits attachment and subsequent infection, presumably by preventing viral interaction with the cell surface, whereas the preS antibody DPSI binds to the preS segment of the envelope protein L and thereby renders the viral particles incompetent for binding to hepatocytes. Analysis of the treated and nontreated primary hepatocytes immunostained for preS (Fig. 2B) largely confirmed the results obtained by immunoblotting of the corresponding cell extracts. Very few infected cells were observed among cells pretreated or concomitantly incubated with suramin, whereas no infected cells were observed in cultures pretreated or concomitantly incubated with DPSI antibody (Fig. 2B, DPSI and suramin). Notably, suramin was slightly less efficient than the preS antiserum in its neutralizing activity to prevent productive infection.

Since preS-specific antibodies and suramin strongly reduced attachment of viral particles and led to a virtually complete abolition of productive DHBV infection in PDHs, they should strongly inhibit viral spread by blocking the second round of infection, provided the extracellular progeny virus alone determines the extent and pattern of viral spread. To test this, we first infected PDHs and incubated them 3 days later with suramin or the preS antisera. At this time point postinfection, productive DHBV infection was established in the hepatocytes, and the first progeny virions started to be released. Three days after the start of treatment, cells were immunostained for preS protein. Immunostaining of infected, untreated PDH cultures for preS revealed that infected cells were not randomly distributed throughout the culture but appeared in clusters of infected cells (Fig. 3). In each of these clusters, one or a few cells were stained prominently (Fig. 3B), surrounded by less strongly labeled cells. The brightly stained cells presumably correspond to the primarily infected ones from which the neighboring cells were infected thereafter. Consistent with this speculation, treatment with neutralizing antibodies prevented this cluster formation: mainly single brightly stained cells could be seen, indicating that progeny virus produced by the primarily infected cells was neutralized by the preS antibody and thereby lost its ability to infect neighboring hepatocytes. Suramin treatment also abolished the formation of clusters of DHBV-infected cells with similar efficiency, implying that released progeny virus needs to bind to the cell surface of adjoining cells for successful infectious entry.

In order to exclude the possibility that the reagents used prevented viral spread not by neutralizing free virions but by acting on intracellular viral replication or impairing release of progeny, we treated the persistently DHBV-producing LMH cell line D2 continuously with suramin or with neutralizing preS antisera for 3 days. Subsequently, cell culture supernatants and cells were harvested and analyzed for viral DNA or

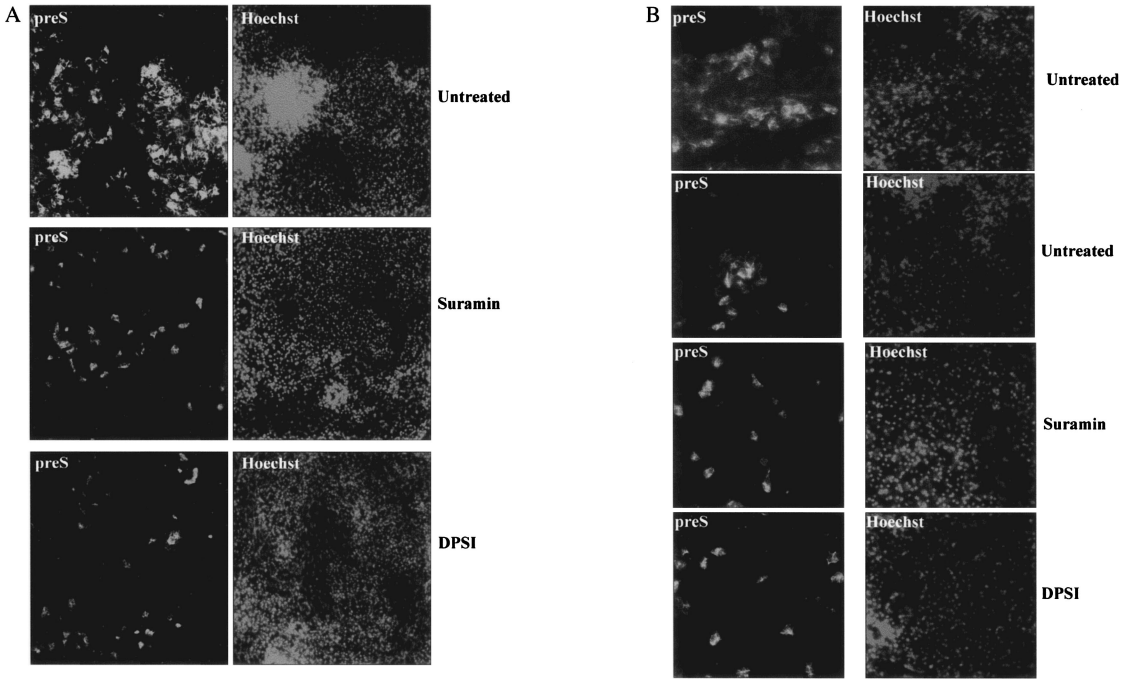

FIG. 3. Neutralizing antibodies and suramin inhibit the spread of DHBV in vitro. PDHs were infected with DHBV. Three days later, suramin or preS antibody DPSI was added to the medium to inhibit the spread mediated by released virions from primarily infected to adjacent cells. The medium was changed daily, and new substances were added. After 3 more days, cells were fixed and stained for preS protein. (A) Lower magnification of the cells showing an overview of the field; (B) higher magnification showing the clusters of infected cells.

preS protein, respectively. No effects on viral secretion (Fig. 4A) or on preS expression (Fig. 4B) were observed, arguing against an effect of these reagents on virus progeny production or release. Analogous experiments performed with naturally infected PDHs also showed no significant changes in the intracellular steady-state levels of viral preS and core protein nor in the cellular protein amount of actin compared to untreated controls (data not shown). Consistent with these results are the data obtained from a cell viability assay with fluorescein diacetate (FDA), an established and sensitive assay for testing the functionality of hepatocyte cultures. After its active uptake, FDA is metabolized and deacetylated. The deacetylated form is fluorescent and can be easily visualized by fluorescence microscopy. Treatment of cultures for 3 days with suramin influenced neither the morphology, the viability, nor the functionality of the hepatocytes. Taken together, these control experiments indicate that suramin interferes with neither the late steps of DHBV replication nor the cellular metabolism of PDHs (data not shown). The observation mentioned above that the treatment of PDHs 2 h after primary infection does not lead to reduced preS levels when measured 3 days thereafter also strongly argues against unspecific effects of both reagents (suramin and preS antisera) on an established infection.

Taken together, these data indicate that the hepadnavirus must first be released from the primarily infected cell and then must bind to the surface of uninfected, adjacent cells for the successful transmission of virus infection. In summary, our data strongly suggest that spread of DHBV during second

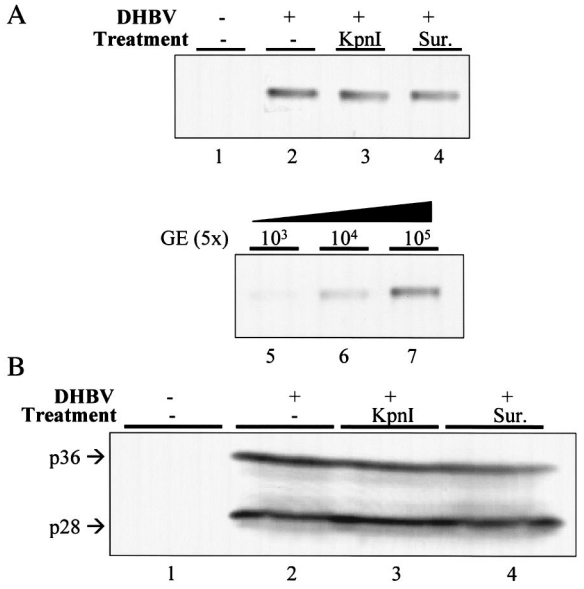

FIG. 4. preS antibodies and suramin do not impair viral secretion or replication. (A and B) Persistently DHBV replicating D2 cells were continuously treated with the preS-specific KpnI antibody or suramin for 3 days. Thereafter, cell culture supernatants and cells were harvested. (A) Analysis of viral DNA in supernatants by PCR. Portions (5 μl) of the supernatants were dissolved in PCR sample buffer and subjected to DHBV DNA-specific PCR. Amplified products were visualized on an ethidium bromide-stained agarose gel after electrophoresis. (B) Analysis of intracellular steady-state levels of preS protein by immunoblotting. Treated cells were lysed in Laemmli buffer and subjected to immunoblot for preS. Arrows point to bands corresponding to the p36 and p28 species of L protein.

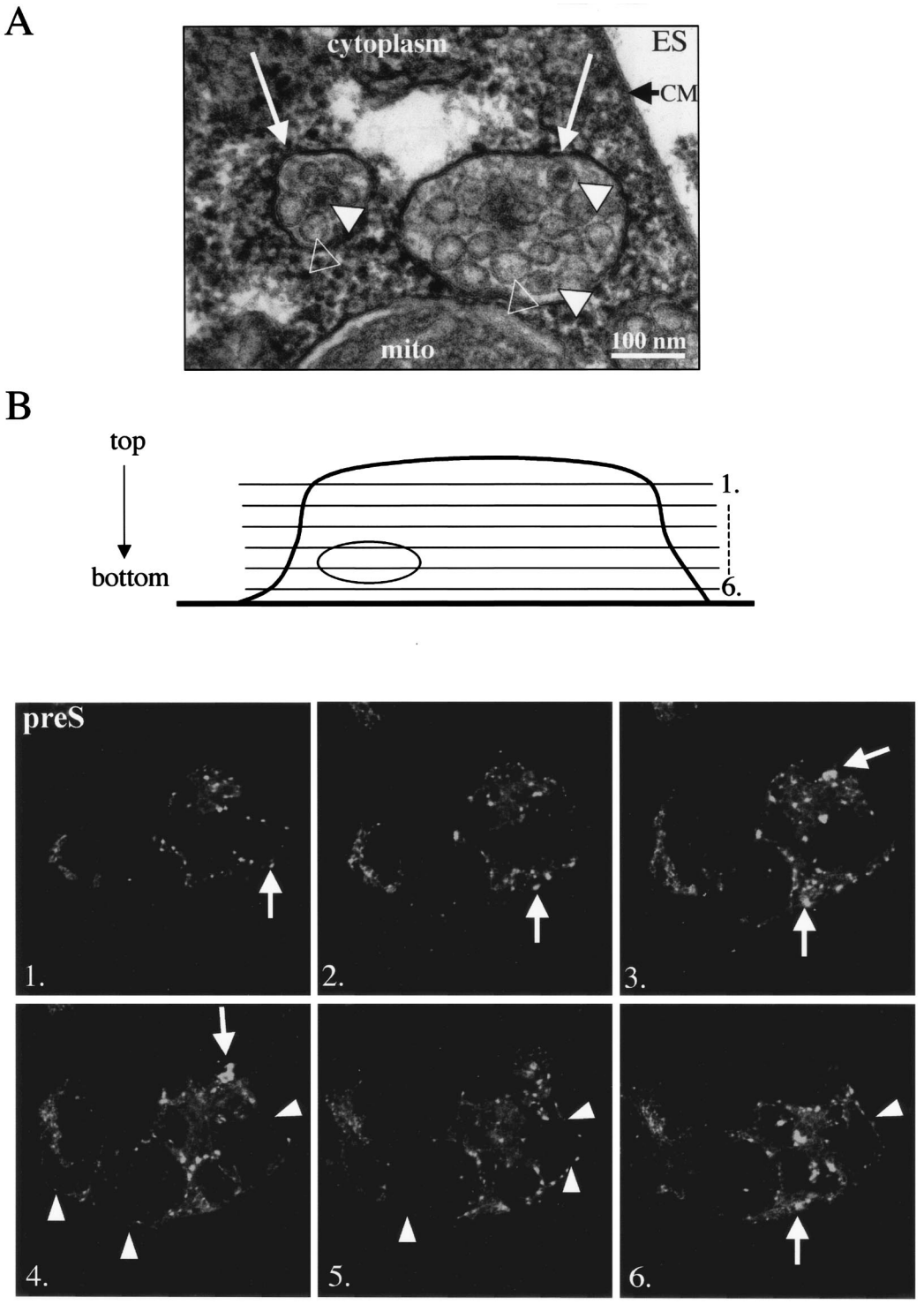

FIG. 5. Electron and confocal microscopic view of intracellular distribution of viral particles in PDHs. (A) Chronically infected PDHs were fixed and processed for electron microscopy 7 days after seeding. A detailed view of the cytoplasmic area of an infected cell is shown. It shows two individual vesicles (arrows) containing virions (solid arrowheads) and numerous subviral particles (open arrowheads). ES, extracellular space; CM, cytoplasmic membrane; mito, mitochondrion. (B) Confocal microscopic analysis of the cellular distribution of L protein in PDHs. Cells grown on coverslips were infected with DHBV, fixed 7 days later, and immunostained for viral L protein and analyzed by confocal laser scanning microscopy. Selected successive sections of the same group of cells (1 to 6, from top to bottom) are shown. The arrowheads indicate cytoplasmic membrane staining; the arrows point to particle-laden vesicles in the hepatocytes.

round of infection requires the release of progeny virus into the extracellular space.

The question remained as to why clusters of infected hepatocytes were observed. This should not be the case if spreading does not occur via cell junctions but is only mediated by progeny viruses eventually released at many random places all along the cellular membrane, from where they can then diffuse or be swept away unhindered into the extracellular environment. A possible explanation for this phenomenon may be that the cluster formation seen in early infection of the cell cultures is due to the polarized egress of virions into intercellular compartments. These putative compartments may hinder the virus from diffusing freely upon secretion. If correct, this assumption would imply that progeny virus is not secreted at random sites along the cell surface but is released preferentially at specific regions. This is certainly conceivable because polarized egress has been recently reported for other viruses, including HIV (for a review, see reference 7), and hepatocytes are well known for their highly polarized organization, including sorting of exocytic vesicles (10, 18). In order to get a first hint as to whether the release of hepadnaviruses occurs by polarized egress, electron microscopic and immunofluorescence staining studies were performed. The ultrastructural analysis of DHBV-infected PDHs by electron microscopy revealed that virions (Fig. 5A, solid arrowheads) and subviral particles (Fig. 5A, open arrowheads) were grouped together in membrane-surrounded cytoplasmic vesicles (Fig. 5A, arrows). In fact, we observed no single free viral particle in the cytoplasm which, if present, should theoretically be transportable much faster than the observed vesicles via cell junctions to neighboring cells. PreS-immunostaining of infected cells showed a punctate pattern consistent with the electron microscopic finding and further indicating that preformed viral particles are subcompartmentalized in vesicular structures in the cytoplasm (Fig. 5B, arrows). Scanning a small colony of infected hepatocytes in horizontal planes of about 0.3-μm thickness by confocal laser scanning microscopy revealed that preS-containing cellular vesicles are not randomly distributed throughout the cytoplasm but preferentially accumulate at the basolateral sites of the cytoplasmic membrane (Fig. 5B). Notably, a prominent staining of the cellular membrane was visible (Fig. 5B, arrowheads). The virus particle-carrying vesicles are presumably transported toward the cellular membrane for release of their cargo and are therefore preferentially found near the cytoplasmic membrane of the infected hepatocyte. Although some particles were found near the apical membrane, most were located near the basolateral membrane. When these viral particle-laden vesicles fuse with the plasma membrane, viruses and subviral particles would be released concomitantly and directed into the basolaterally located intercellular space. This may well hamper free diffusion and promote preferential formation of clusters of infected cells.

Taken together, our data strongly suggest that hepadnaviruses mainly or exclusively spread via extracellular progeny to adjacent cells through polarized egress into intercellular compartments. Insights into the spreading mode of hepadnaviruses and the identification of substances that prevent the viral spread may provide additional opportunities to develop urgently needed new strategies that allow efficient treatment of chronic hepatitis B infection.

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